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β-catenin is a critical signaling molecule that participates in differentiation and proliferation pathways. β-catenin binds the HMG-box transcription factor LEF-1, to directly regulate gene transcription. The tumor suppressor adenomatous polyposis coli (APC) gene product has been reported to associate with β-catenin and effect its down-regulation by an unknown mechanism. We hypothesize that APC helps target β-catenin to proteasomal degradation. We tested our hypotheses *in vivo* by transient transfections of colon cancer cells with various APC deletion constructs, followed by treatment with proteasome-specific inhibitors. The LEF-reporters TOPFLASH and FOPFLASH were used to demonstrate a role for APC in regulating β-catenin-LEF signaling via the ubiquitin-proteasome pathway. Surprisingly, lithium (that inhibits GSK3β also) was found not to reverse the ability of APC to down-regulate LEF signaling.

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INTRODUCTION

β-catenin is a signaling molecule

β-catenin is a multifunctional protein that primarily helps link the cadherins (at the adherens junctions) to the cytoskeleton. However, β-catenin is also a crucial signaling molecule that participates in differentiation and proliferation pathways. The *wnt* signaling pathway, known to reverse contact inhibition in mouse mammary cells *in vitro* and to cause mammary cancer in mice (7), results in increased levels of cytoplasmic β-catenin (8). *Wnt*-1 stimulation results in decreased activity of glycogen synthase kinase (GSK)-3β, that normally phosphorylates the tumor suppressor adenomatous polyposis coli (APC) gene product (8,9). When APC is not phosphorylated, it leads to the stabilization of β-catenin. The stable β-catenin interacts with the transcriptional activators Lymphocyte Enhancer-binding Factor/ T Cell Factor (LEF/TCF) (10). The β-catenin-TCF/LEF complex translocates to the nucleus and effects gene expression (1,2) . The genes activated may include those that stimulate proliferation or antagonize apoptosis (11,12). And finally, stable forms of β-catenin by themselves are oncogenic (3,12,13). These observations strongly point towards the stability of cytoplasmic β-catenin as a "smoking gun" (12) linking cell adhesion and tumorigenesis. Thus, a strategy of down-regulating β-catenin could constitute a potential way of treating breast cancer.

In this study, we investigate the APC mediated regulation of β-catenin stability and β-catenin-LEF signaling.

β- Catenin and breast cancer

Cells touch one another through a number of different surface molecules; among the most intriguing are the cadherins and their associated proteins (14). These proteins, in addition to maintaining adhesion of adult tissues, via the adherens junctions, are critical during development and tumorigenesis (15). Cadherin function has been shown to depend on several associated proteins, namely; α, β, and (plakoglobin) γ catenin (16). These molecules, link cadherins to the actin cytoskeleton and are probably involved in relaying cadherin-mediated-contact signals (17). The β-catenin/cadherin association requires serine phosphorylation of the cadherin molecule (17). β–catenin is itself a substrate for tyrosine phosphorylation and can also act as a link between Growth factor receptors (such as the EGFR) and the adherens junction complex (18,19). Mutation of the β-catenin gene in mice, by homologous recombination, results in embryonal lethality. When the expression of E-cadherin and the catenins was analyzed in human breast carcinomas, lobular breast carcinomas showed disturbances of E-cadherin and catenins in a high frequency of cases (20). In ductal breast carcinomas (where E-cadherin is often unchanged), a high frequency of cases showed disturbance of alpha- and/or gamma-catenin expression. 50 % of cases with defects in E-cadherin and catenins had lymph node metastasis, whereas this number was low in cases with undisturbed cadherin/catenin expression (20).

A truncated stable form of β-catenin itself acts as an oncogene (9). The phosphorylation state of β-catenin can also influence the transformed phenotype (19,21). Further, cytoplasmic β-catenin associates with the tumor suppressor adenomatous polyposis coli (APC) gene product (19). Over-expression of APC results in the cell cycle being blocked at the G1/S boundary (19). Recent evidence indicating that the tumor suppressor effects of APC are dependent upon its

ability to destabilize β-catenin, strongly argue the significance of β-catenin in the control of cell proliferation (5,22).

SPECIFIC AIMS (Year 3)

Aim 1. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) regulates β-catenin-LEF signaling

Aim 2. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) can down-regulate wtβ-catenin- but not a non-ubiquitinatable S37A mutant β-catenin-induced LEF signaling

Aim 3. To test the hypothesis that Lithium, an inhibitor of GSK3β also, represses the ability of the tumor suppressor adenomatous polyposis coli (APC) to down-regulate β-catenin-LEF signaling

METHODS

Aim 1. To test the hypotheis that APC regulates β-catenin-LEF signaling

LEF-Luciferase Reporter Assays (44): Cells were seeded in 12 well-plates at 1 x 10^5 cells per well. The following day, cells were transiently transfected with 1 μg of APC constructs, 0.4 μg of the LEF-reporter pTOPFLASH (with optimal LEF binding sites) (44), 0.008 μg pCMV-Renilla Luciferase (Promega), per well, using Lipofectamine-Plus reagent according to manufacturers instructions (GIBCO-BRL). In experiments designed to monitor the effect of APC on β-catenin protein, 0.3μg FLAG-tagged wt β-catenin was co-transfected with 0.6μg empty vector or APC constructs. 12 hr post-transfection, cells were treated with indicated levels of the inhibitors for 12 hr. Luciferase activity was monitored (24 hr post-transfection) using the Dual-Luciferase Assay System (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments using the constitutively expressed Renilla luciferase. The specificity of APC mediated effects on LEF-reporters were confirmed using pFOPFLASH which harbors mutated LEF binding sites, and an unrelated AP-1 reporter.

To monitor changes in β-catenin protein levels, SW480 cells were transiently transfected with FLAG-tagged wt β-catenin and empty vector or APC25. The co-transfection of tagged-β-catenin facilitated selective analysis of transfectants only. 12 hr. post-transfection, cells were treated with 10 μM ALLN or DMSO (vehicle). 12 hr. later the cells were lysed in NP-40 lysis buffer. Cell lysates were Western blotted using anti-FLAG (Kodak) and anti-GAPDH antibodies (Research Diagnostics).

Aim 2. To test the hypothesis that APC down-regulates wt β-catenin- but not the non-ubiquitinatable, S37A mutant β-catenin- induced LEF signaling

Wt or S37A mutant β-catenin (45) constructs were co-transfected with empty vector or APC 25 and the LEF-reporters, into SW480 cells. Reporter assays were performed as described under Aim 1.

Aim 3. To test the hypothesis that Lithium, an inhibitor of GSK3β also, can repress the ability of the tumor suppressor adenomatous polyposis coli (APC) to down-regulate β-catenin-LEF signaling

The colon cancer cell line SW480 was transfected with LEF-reporters and empty vector or wtAPC. Following transfection, cells were treated with 10, 20, or 40 mM LiCl or NaCl (46). Reporter-gene assays were performed as described under Aim 1.

RESULTS

Aim 1. To test the hypotheis that the tumor suppressor adenomatous polyposis coli (APC) regulates β-catenin-LEF signaling

Fig. 1 (addenda) shows that the APC mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN, Lactacystin, and MG-132, but not DMSO (vehicle) alone or ALLM. The proteasomal inhibitor ALLN reverses the APC mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner (Fig. 2). In the same experiment β-catenin protein levels were monitored by immunoblot, to verify that changes in APC mediated β-catenin-LEF signaling were indeed due to changes in cytoplasmic β-catenin (Fig. 3). These observations demonstrate that APC mediated down-regulation of β-catenin-LEF signaling requires functional proteasomes.

Aim 2. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) can down-regulate WT but not the non-ubiquitinatable, S37A mutant β-catenin induced LEF signaling.

Mutation of a single serine residue (S37A) within the ubiquitination targeting sequence (UTS) prevents β-catenin ubiquitination (45). Non-ubiquitinated β-catenin is not a substrate for the proteasome and thus accumulates in the cytoplasm. The S37 residue of β-catenin is found mutated in a variety of cancer cell lines, leading to stabilization of the protein. If APC regulates β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation, then it should not be able to regulate the non-ubiquitinatable S37A mutant β-catenin induced LEF signaling.

Wt or S37A mutant β-catenin constructs were co-transfected with empty vector or APC 25 and the LEF-reporters, into SW480 cells. APC down-regulates wt β-catenin- but not the S37A mutant β-catenin- induced LEF signaling (Fig. 4). These observations further suggest that APC regulates β-catenin-LEF signaling through a potential role in the ubiquitin-proteasomal degradation of β-catenin.

Aim 3. To test the hypothesis that Lithium, an inhibitor of GSK3β also, alters the ability of the tumor suppressor adenomatous polyposis coli (APC) to down-regulate β-catenin-LEF signaling.

Stambolic et al., (46) have convincingly established that physiologically effective concentrations of Li^+ specifically and reversibly inhibit GSK-3β activity *in vitro* and *in vivo*, and that Li^+ can mimic the effects of Wnt signaling on β-catenin in mammalian cells. Treatment of the breast cancer cell lines SKBR3 and HBL100 with lithium results in the accumulation of the cytoplasmic, signaling pool of β-catenin (45). The exact mechanism by which inhibition of GSK-3β leads to stabilization of β-catenin is unknown.

We tested the hypothesis that Li^+ can inhibit the ability of APC to down-regulate β-catenin - LEF signaling. The colon cancer cell line SW480 was transfected with empty vector or wtAPC, and treated with 10, 20, or 40 mM LiCl or NaCl. Fig. 5 shows that lithium does not significantly alter the ability of wt APC to down-regulate LEF-reporter activity. In light of recent additions to the

Wnt signaling pathway (e.g. axin) (47), the effect of lithium and inhibition of GSK-3β on β-catenin-LEF signaling and the precise role of APC in this process need to be evaluated in further detail.

CONCLUSIONS

1. APC mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors.
2. The APC induced reduction in LEF signaling and the inhibition of this reduction by proteasomal inhibitors are paralleled by changes in β-catenin protein.
3. APC down-regulates wt β-catenin- but not the non-ubiquitinatable S37A mutant β-catenin-induced LEF signaling.
4. Lithium, an inhibitor of GSK3β also, does not significantly alter the ability of wt APC to down-regulate β-catenin-LEF signaling.

BIBLIOGRAPHY

1. Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. Constitutive transcriptional activation by a b-catenin-Tcf Complex in $APC^{-/-}$ colon carcinoma. Science, *275*: 1784-1787, 1997.

2. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. Activation of b-catenin/Tcf signaling in colon cancer by mutations in b-catenin or APC. Science, *275*: 1787-1790, 1997.

3. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. Stabilization of b-catenin by genetic defects in melanoma cell lines. Science, *275*: 1790-1792, 1997.

4. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. U. S. A. *92*: 3046-3050, 1995.

5. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. Binding of GSK3 beta to the APC-beta-catenin complex and regulation of complex assembly. Science, *272*: 1023-1026, 1996.

6. Vleminckx, K., Wong, E., Guger, K., Rubinfeld, B., Polakis, P., and Gumbiner, B. Adenomatous polyposis coli tumor suppressor protein has signaling activity in xenopus laevis embryos resulting in the induction of an ectopic dorsoanterior axis. J. Cell Biol. *136*: 411-420, 1997.

7. Moser, A.R., Mattes, E.M., Dove, W.F., Lindstrom, M.J., Haag, J.D., and Gould, M.N. ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. Proc. Natl. Acad. Sci. U. S. A. *90*: 8977-8981, 1993.

8. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. Mol. Cell Biol. *16*: 2128-2134, 1996.

9. Hinck, L., Nelson, W.J., and Papkoff, J. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. J. Cell Biol. *124*: 729-741, 1994.

10. Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. Functional interaction of b-catenin with the transcription factor LEF-1. Nature, *382*: 638-642, 1996.

11. Peifer, M. Regulating cell proliferation: as easy as APC. Science, *272*: 974-9751996.

12. Peifer, M. Beta Catenin as oncogene: The smoking gun. Science, *275*: 1752-1753, 1997.

13. Whitehead, I., Kirk, H., and Kay, K. Expression cloning of oncogenes by retroviral transfer of cDNA libraries. Mol. Cell Biol. *15*: 704-710, 1995.

14. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science, *251*: 1451-1455, 1991.

15. Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol. *113*: 173-185, 1991.

16. Gumbiner, B.M. and McCrea, P.D. Catenins as mediators of the cytoplasmic functions of cadherins. J. Cell Sci. Suppl. *17*: 155-158, 1994.

17. Cowin, P. and Burke, B. Cytoskeleton-membrane interactions. Curr. Opin. Cell Biol. *8*: 56-65, 1996.

18. Hoschuetzky, H., Aberle, H., and Kemler, R. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J. Cell Biol. *127*: 1375-1380, 1994.

19. Shibamoto, S., Hayakawa, K., Takeuchi, T., Hori, N., Oku, K., Miyazawa, N., Kitamura, M., Takeichi, M., and Ito, F. Tyrosine phosphorylation of beta catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adh. Commun. *1*: 295-305, 1994.

20. Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M., and Birchmeier, W. Loss of Epithelial Differentiation and Gain of Invasiveness Correlates with Tyrosine Phosphorylation of the E-Cadherin/B-Catenin Complex in Cells Transformed with a Temperature-Sensitive v-SRC Gene. JCB, *120*: 757-766, 1993.

21. Sommers, C.L., Gelmann, E.P., Kemler, R., Cowin, P., and Byers, S.W. Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast cancer cell lines. Cancer Res. *54*: 3544-3552, 1994.

22. Munemitsu, S., Albert, I., Rubinfeld, B., and Polakis, P. Deletion of an amino-terminal sequence stabilizes b-catenin in vivo and promotes hyperphosphorylation of the adenomatous polyposis coli tumor suppressor protein. Mol. Cell Biol. *16*: 4088-4094, 1996.

23. Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B.M. Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. J. Cell Biol. *128*: 959-968, 1995.

24. McCrea, P.D., Brieher, W.M., and Gumbiner, B.M. Induction of a secondary body axis in Xenopus by antibodies to beta-catenin. J. Cell Biol. *123*: 477-484, 1993.

25. Reise, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S., Grosschedl, R., and Bienz, M. LEF-1, a nuclear factor co-ordinating signaling inputs from wingless and decapentaplegic. Cell, *88*: 777-787, 1997.

26. Su, L., Vogelstein, B., and Kinzler, K.W. Association of the APC tumor suppressor protein with catenins. Science, *262*: 1734-1737, 1993.

27. Baeg, G., Matsumine, A., Kuroda, T., Bhattacharjee, R.N., Miyaashiro, I., Toyoshima, K., and Akiyama, T. The tumor suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. EMBO J, *14*: 5618-5625, 1995.

28. Ciechanover, A. The ubiquitin-proteasome pathway. Cell, *79*: 13-21, 1994.

29. Orian, A., Whiteside, S., Israel, A., Stancovski, I., Schwartz, A.L., and Ciechanover, A. Ubiquitin-mediated processing of NF-kappa B transcriptional activator precursor p105. Reconstitution of a cell-free system and identification of the ubiquitin-carrier protein, E2, and a novel ubiquitin-protein ligase, E3, involved in conjugation. J. Biol. Chem. *270*: 21707-21714, 1995.

30. Stancovski, I., Gonen, H., Orian, A., Schwartz, A.L., and Ciechanover, A. Degradation of the proto-oncogene product c-Fos by the ubiquitin proteolytic system in vivo and in vitro: identification and characterization of the conjugating enzymes. Mol. Cell Biol. *15*: 7106-7116, 1994.

31. Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell, *75*: 495-505, 1995.

32. Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, *78*: 761-771, 1994.

33. Grenfell, S.J., Trausch Azar, J.S., Handley Gearhart, P.M., Ciechanover, A., and Schwartz, A.L. Nuclear localization of the ubiquitin-activating enzyme, E1, is cell-cycle-dependent. Biochem. J. *300*: 701-708, 1993.

34. Treier, M., Staszewski, L.M., and Bohmann, D. Ubiquitin-dependent c-jun degradation in vivo is mediated by the delta domain. Cell, *78*: 787-798, 1995.

35. Shkedy, D., Gonen, H., Bercovich, B., and Ciechanover, A. Complete reconstitution of conjugation and subsequent degradation of the tumor suppressor protein p53 by purified components of the ubiquitin proteolytic system. FEBS Lett. *348*: 126-130, 1994.

36. Gonen, H., Stancovski, I., Shkedy, D., Hadari, T., Bercovich, B., Bengal, E., Mesilati, S., Abu Hatoum, O., Schwartz, A.L., and Ciechanover, A. Isolation, characterization, and partial

purification of a novel ubiquitin-protein ligase, E3. Targeting of protein substrates via multiple and distinct recognition signals and conjugating enzymes. J. Biol. Chem. *271*: 302-310, 1996.

37. Blumenfeld, N., Gonen, H., Mayer, A., Smith, C.E., Siegel, N.R., Schwartz, A.L., and Ciechanover, A. Purification and characterization of a novel species of ubiquitin-carrier protein, E2, that is involved in degradation of non-"N-end rule" protein substrates. J. Biol. Chem. *269*: 9574-9581, 1994.

38. Zhang, M., Zou, Z., Maass, N., and Sager, R. Differential expression of elafin in human normal mammary epithelial cells and carcinomas is regulated at the transcriptional level. Cancer Res. *55*: 2537-2541, 1995.

39. Stampfer, M.R., Pan, C.H., Hosoda, J., Bartholomew, J., Mendelsohn, J., and Yaswen, P. Blockage of EGF receptor signal transduction causes reversible arrest of normal and immortal human mammary epithelial cells with synchronous reentry into the cell cycle. Exp. Cell Res. *208*: 175-188, 1993.

40. Urbani, L., Sherwood, S.W., and Schimke, R.T. Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. Exp. Cell Res. *219*: 159-168, 1995.

41. Igawa, M., Rukstalis, D.B., Tanabe, T., and Chodak, G.W. High levels of nm23 expression are related to cell proliferation in human prostate cancer. Cancer Res. *54*: 1313-1318, 1994.

42. Levenson, V. and Hamlin, J.L. A general protocol for evaluating the specific effects of DNA replication inhibitors. Nucleic Acids. Res. *21*: 3997-4004, 1993.

43. Brandeis, M. and Hunt, T. The proteolysis of mitotic cyclins in mammalian cell persists from the end of mitosis until the onset of S-phase. EMBO. J. *15*: 5280-5289, 1996.

44. Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell, *88*: 789-99, 1997.

45. Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M., and Byers, S.W. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J. Biol. Chem. *272*: 24735-24738, 1997.

46. Stambolic, V., Ruel, L., and Woodgett, J.R. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr. Biol. *6*: 1664-1668, 1996.

47. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., Kikuchi, A. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J. Biol. Chem. *273*: 10823-10826, 1998.

ADDENDA

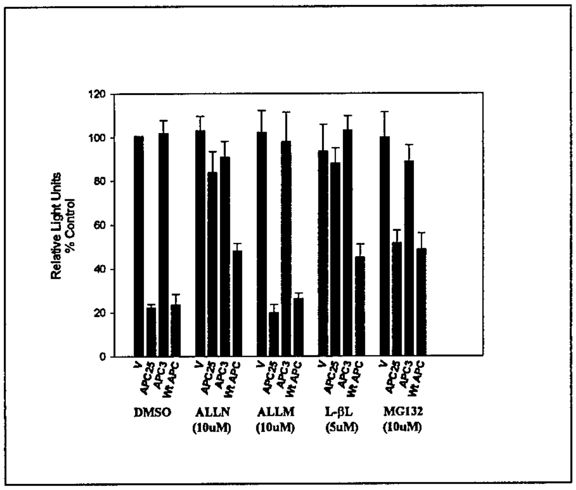

Figure 1. APC mediated down-regulation of β-catenin-LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs and treated with proteasomal inhibitors ALLN, Lactacystin-β *lactone*, and MG-132, or with DMSO (vehicle) and control-peptide ALLM. β-catenin-LEF signaling was assayed using the LEF-reporters pTOPFLASH and pFOPFLASH.

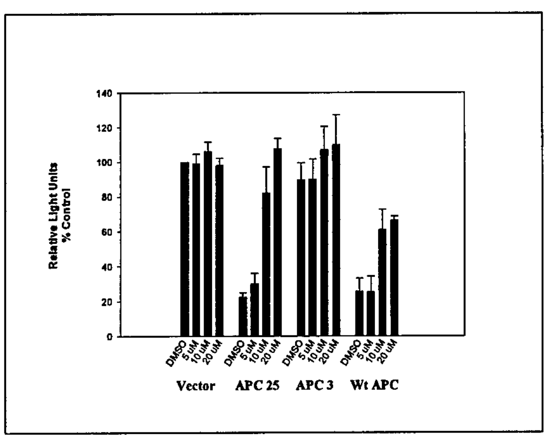

Figure 2. APC mediated down-regulation of β-catenin-LEF signaling is reversed by the proteasomal inhibitor ALLN, in a dose-dependent manner.

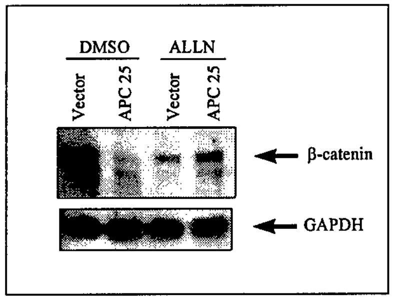

Figure 3. The APC induced reduction in LEF signaling and the inhibition of this reduction by the proteasomal inhibitor ALLN are paralleled by changes in β-catenin protein. SW480 cells were transiently transfected with FLAG-tagged wt β-catenin and empty vector or APC25. 12 hr. post-transfection, cells were treated with 10 μM ALLN or DMSO (vehicle). 12 hr. later the cells were lysed in NP-40 lysis buffer. Cell lysates were Western blotted using anti-FLAG and anti-GAPDH antibodies.

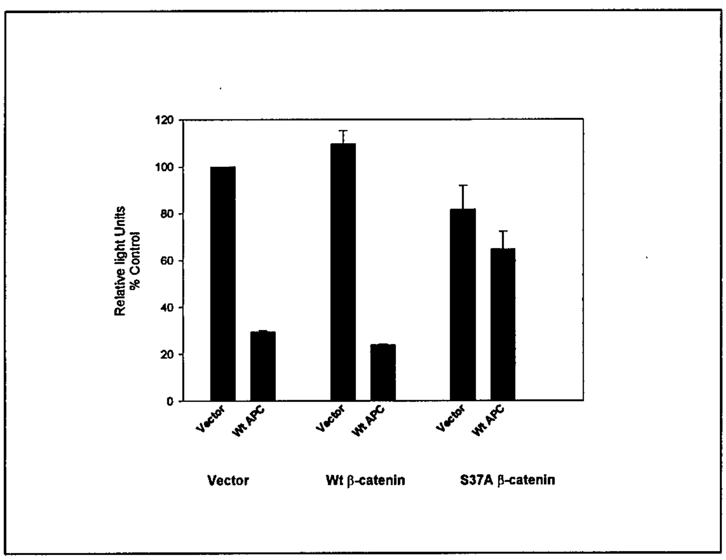

Figure 4. APC down-regulates wt β-catenin- but not the non-ubiquitinatable S37A mutant β-catenin- induced LEF signaling

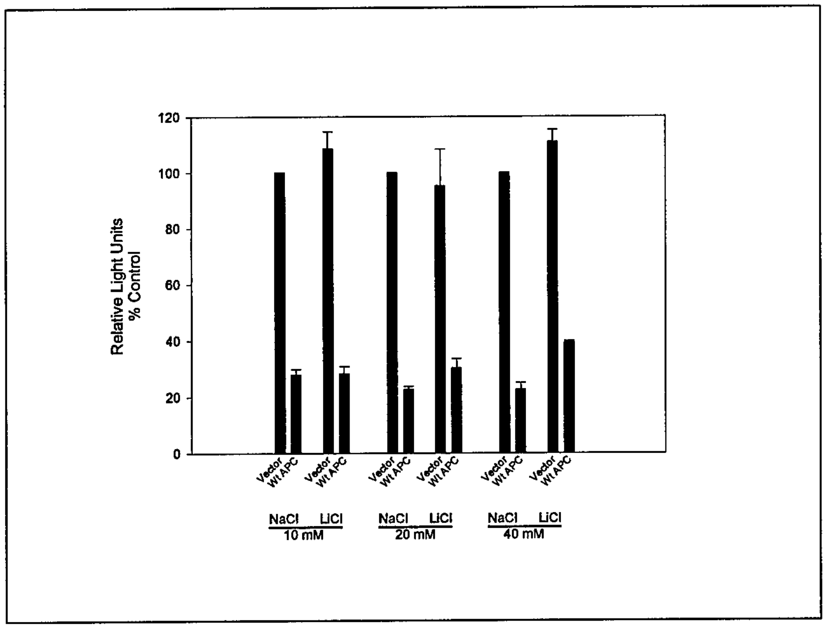

Figure 5. Lithium, an inhibitor of GSK3β also, does not inhibit the ability of APC to down-regulate β-catenin-LEF signaling.